Food Chemistry 329 (2020) 127178

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Betacyanins from *Gomphrena globosa* L. flowers: Incorporation in cookies as natural colouring agents

Custódio Lobo Roriz[a,b], Sandrina A. Heleno[a,*], Márcio Carocho[a], Paula Rodrigues[a], José Pinela[a], Maria Inês Dias[a], Isabel P. Fernandes[a], Maria Filomena Barreiro[a], Patricia Morales[b], Lillian Barros[a,*], Isabel C.F.R. Ferreira[a]

[a] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
[b] Dpto. Nutrición y Ciencia de los Alimentos. Facultad de Farmacia, Universidad Complutense de Madrid (UCM), Madrid, Spain

ARTICLE INFO

Keywords:
Gomphrena globosa L.
Betacyanins
Natural colourants
Pastry industry
Stability

ABSTRACT

A betacyanin rich extract was obtained from the flowers of *Gomphrena globosa* L. by ultrasound-assisted extraction and dried either by lyophilization or spray-drying, was tested as a natural colourant in cookies and compared to a commercial colourant. The extracts were characterized in terms of betacyanin content and antioxidant potential. The effects of the colourants incorporation in the cookies were assessed through proximate composition, soluble sugars, fatty acids, color, texture and microbial load, over a shelf life of 30 days. Considering all the assays and analyzing the results through a 2-way analysis of variance, the cookies incorporated with spray-dried colourant showed the most intense pink coloration while cookies incorporated with lyophilized extract lost less color intensity over time. Thus, betacyanin extracts have potential as pink natural alternatives to synthetic colourants in the food industry.

1. Introduction

Natural food additives have been used in the food industry for a long time (Tomaska & Brooke-Taylor, 2014). Nevertheless, these natural additives present some stability problems as is the case of natural colourants (Wu et al., 2020), which are sensitive to temperature, pH, light and storage conditions, leading to the assumption that these additives are not viable for industrial applications (Leong et al., 2018). The synthetic counterparts present higher stability under the same conditions as well as higher colouring capacity and maintenance (Martins, Roriz, Morales, Barros, & Ferreira, 2016). Recent scientific studies have shown that artificial additives have several side effects and are often associated with toxicity problems (Zhang et al., 2020), such as hyperactivity and allergies (Kumar, Singh, Sharma, & Kishore, 2019). Aware of these evidences, the regulatory agencies, namely the European Food Safety Authority (EFSA) and the Food and Drug Administration of the United States of America (FDA) established several restrictions on the use of these additives (Carocho, Morales, & Ferreira, 2015). Together with these scientific facts, consumers also started to show extra care about their health and started to seek for safer and natural additives, causing an enormous pressure to the industry and scientific community (Roriz, Barreira, Morales, Barros, & Ferreira, 2018). This fact, associated with current food trends, where visual traits are an important attribute (Stich, 2016), together with health benefits (Martins, Sentanin, & De Souza, 2019), a new era of scientific challenges focused on the exploitation of natural resources for industrial applications, with safer and health-promoting agents (Maqsood, Adiamo, Ahmad, & Mudgil, 2020). These functional products should provide consumers with new experiences and, at the same time, be able to meet nutritional needs, showing preventive disease effects (Roriz et al., 2018). All of these different functions can be obtained from natural sources due to the richness in bioactive compounds, able to provide health benefits and at the same time act as food preservatives, colouring agents among other properties (Majerska, Michalska, & Figiel, 2019).

One of the most important agents in the food industry are colourants, which are especially responsible for the attractiveness of foods. In this particular case, there are already some natural colourants approved by EFSA, namely carotenoids, chlorophylls, anthocyanins and betalains (Martins et al., 2016). The latter, for instance, are chromoalkaloids derived from tyrosine, divided into two subgroups, the betaxanthins that present a yellow/orange colour and the betacyanins, with colours between pink and violet (Martins, Roriz, Morales, Barros, & Ferreira, 2017). Betacyanins are very similar to anthocyanins, regarding their

* Corresponding authors.
E-mail addresses: sheleno@ipb.pt (S.A. Heleno), lillian@ipb.pt (L. Barros).

https://doi.org/10.1016/j.foodchem.2020.127178
Received 7 February 2020; Received in revised form 22 May 2020; Accepted 27 May 2020
Available online 30 May 2020
0308-8146/

colour, but showing a stronger colouring power (Roriz, Barros, Prieto, Morales, & Ferreira, 2017). The preferable natural source to extract this class of compounds from is beetroot (*Beta vulgaris* L.) although there are also flowers and fruits that can be considered as good sources of this natural colourant (Calogero et al., 2012; Melgar et al., 2017; Roriz, Barros, Prieto, Barreiro et al., 2017). Since natural colourants became a global hot topic, new extraction, purification and stabilization procedures needed to be developed (Corrêa et al., 2019) to guarantee the viability of these colourants as candidates for effective applications at the industrial level (Roriz et al., 2018; Roriz, Barros, Prieto, Barreiro et al., 2017).

Gomphrena globosa L. flowers have purple colour and are a rich source of betalains, namely betacyanins with strong colouring capacity as described in a recent work, where the incorporation of *G. globosa* extracts in ice cream showed promising results regarding their colouring potential (Roriz et al., 2018).

Thus, the objective of the present work was to evaluate the potential of *G. globosa* flowers as a source of betalains and their viability as colouring agents in the food industry, namely in pastry products. The extracts were obtained by ultrasound-assisted extraction (UAE) and further dried through spray-drying and lyophilization techniques. The stability of these extracts after incorporation into cookies, was studied, namely the centesimal composition, chemical and physical parameters, over a shelf-life of 30 days. All the performed assays with cookie formulations were compared with samples incorporated with a widespread commercial colourant (E162).

2. Material and methods

2.1. Plant samples

Gomphrena globosa L. plants were acquired from Ervital, a company established in a mountain region of great biodiversity, Castro D'Aire, Portugal. Ervital has a wide certified collection of vegetable materials from different origins and applies sustainable harvesting of spontaneous local species, and organic farming of exogenous species. The botanical identification was made by the botanical expert responsible for the collection of medicinal plants in the herbarium of Escola Superior Agrária (BRESA), Polytechnic Institute of Bragança (Trás-os-Montes, Portugal). Subsequently, the plants were subjected to a mechanical treatment to separate the coloured parts of the flower (intense purple-pink colour), obtaining the pigmented floral parts (bracts and bracteoles) from the inflorescences, as described by Roriz, Barros, Prieto, Barreiro et al. (2017).

2.2. Preparation of the betacyanin's rich extracts

G. globosa extracts rich in betacyanins were obtained by UAE (QSonica sonicators, model CL-334, Newtown, CT, USA), working at 500 W, for 22 min, using water (treated in a Milli-Q water purification system, TGI Pure Water Systems, Greenville, SC, USA) as the preferred extraction solvent and a liquid-to-solid ratio of 5 g/L, as described previously by Roriz et al. (2018). The extracts were further dried using two different methodologies: i) lyophilization (FreeZone 4.5, Labconco, Kansas City, MO, USA) and ii) spray-drying, to evaluate the impact of these technologies towards the stability of the extracts. In the lyophilization process, the extracts were placed in appropriate containers and frozen at −80 °C through a fast-freezing process, in order to avoid compromising the stability of the colouring compounds. After freezing, the extracts were placed in the lyophilizer at a constant temperature of −45 °C and 0.04 mBar of pressure until the samples were completely dried (4 days). For spray-drying, the samples were dried using 20% of maltodextrin (w/w), a drying adjuvant (Molina et al., 2019). The percentage of maltodextrin (20%, w/w) was relative to the total solids content of the extract sample to be spray-dried, being established in previous studies (Molina et al., 2019). The solutions containing the betacyanins and maltodextrin were prepared immediately before atomization. Briefly, the extracts were mixed with maltodextrin and further homogenized by stirring for 10 min at room temperature, using a stirring plate and a magnetic bar. The used spray-drying equipment was a Mini Spray Dryer B-290 Büchi (Flawil, Switzerland) programmed in normal operation mode (nozzle diameter: 0.7 mm; atomized volume: 200 mL, solids content < 33%). The used operation conditions were established according to previous works using this drying co-adjuvant (inlet temperature 140 °C, outlet temperature 72 °C, aspiration 90% and pump 20% (6 mL/min)) (Molina et al., 2019). The collected dry samples were kept in sterile flasks protected from light (4 °C) until further analysis. The overall yield was estimated as the ratio between the weight of recovered powder (dry basis) and the weight of the initial solids in the atomized solution (dry basis). In the lyophilization process, a non-powdered and heterogeneous sample was obtained displaying a dark purple colour, while the spray-drying technique rendered a homogeneous and shiny powder displaying a deep purple-pink hue.

2.3. Betalains quantification and identification

Betacyanin were profiled using a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) couple to a DAD (using 530 nm as the preferred wavelength) and to a mass spectrometer working in positive mode (Linear Ion Trap LTQ XL, Thermo Finnigan, San Jose, CA, USA) and equipped with an ESI source, following a procedure previously reported (Melgar et al., 2017). The separation was carried out in an AQUA® (Phenomenex) reverse phase C_{18} column (5 μm, 150 × 4.6 mm i.d) operating at 35 °C. The betacyanin's identification was performed by comparing the obtained information with available data reported in the literature giving a tentative identification. For quantitative analysis, a calibration curve using an isolated compound gomphrenin III (isolated from *Gomphrena globosa* L.) was constructed based on the UV signal ($y = 14670x - 19725$, $R^2 = 0.9997$) (Roriz et al., 2017). The results of betacyanins were expressed as mg per g of extract.

2.4. Evaluation of the anti-haemolytic activity

The antioxidant capacity of the extracts was evaluated by the oxidative haemolysis inhibition assay (OxHLIA). An erythrocyte solution (2.8%, v/v; 200 μL) was mixed with 400 μL of either extracts dissolved in phosphate-buffered saline (PBS), having one control with only PBS solution, or water (for complete haemolysis). After a pre-incubation period of 10 min with shaking at 37 °C, 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) (200 μL, 160 mM in PBS, from Sigma-Aldrich) was added, and the optical density (690 nm) measured every 10 min using a microplate reader (Bio-Tek Instruments, ELX800) until complete haemolysis (Lockowandt et al., 2019). Trolox was the positive control. The results were expressed as IC_{50} values (μg/mL) at a Δt of 60 min, representing the concentration required to keep 50% of the erythrocyte population intact for 60 min.

2.5. Preparation of the cookies with the colouring agents

To prepare the cookies, 450 g of butter (pasteurized butter Continente®; composition per 100 g: 3016 KJ, or 733 Kcal energetic value; 81 g of lipids of which 58 g were saturated; 0.6 g of carbohydrates, being the majority sugars; 0.5 g of protein; and 1.8 g of salt) was mixed with 540 g of sugar (granulated white sugar Continente®; composition per 100 g: 1700 KJ, or 400 Kcal energetic value; 0 g of lipids; 100 g of carbohydrates of which 100 g were sugars; 0 g fibres; 0 g of protein; and 0 g of salt). Then, 840 g of flour (self-raising wheat flour Continente®; composition per 100 g: 1421 KJ, or 333 Kcal energetic value; 1 g of lipids of which 0.3 g were saturated; 71 g of carbohydrates of which 2 g were sugars; 3 g of fibres; 9 g of protein; and 2 g of salt), 20 g of yeast powder (yeast powder Continente®; composition per

100 g: 589 KJ, or 139 Kcal energetic value; 0.8 g of lipids of which < 0.1 g are saturated; 32.5 g of carbohydrates of which < 0.1 g are sugars; 0.1 g fibres; 0.5 g of protein; and 31.1 g of salt), 50 g of water and 100 g of vegetable oil (cooking oil Continente®; composition per 100 mL: 3397 KJ, or 826 Kcal energetic value; 92 g of lipids of which 10 g are saturated; 0 g of carbohydrates; 0 g of protein; and 0 g of salt) mixed with a stand mixer (Food Processor SKM 550 A1, SilverCrest, Hamburg, Germany). The cookie dough was divided into four equivalent parts, and identified as: i) control (cookie dough without colouring agents); ii) cookie dough coloured with E162 (commercial natural food colourant; 2 g, i.e. ≈4 mg/100 g cookie dough); iii) cookie dough coloured with lyophilized *G. globosa* extract (1 g, i.e. ≈2 mg/100 g cookie dough); iv) cookie dough coloured with spray-dried *G. globosa* extract 1 g, i.e. ≈2 mg/100 g cookie dough). For the batches where the colourant was added, the dough was continuously kneaded until the dough color presented was homogeneous. For each batch, the cookie dough was divided into approximately 12 g balls, on a tray lined with parchment paper and baked for 25 min at ≈ 140 °C. After baking, the cookies were separated to be analysed through at four different storage times, using 10 cookies per time. Prior to analysis, all cookie samples were lyophilized, finely crushed and analyzed (in triplicate), immediately after preparation, and at three more sampling times (7; 15; and 30 days of storage). Cookies were stored at room temperature and packed in a sealed plastic bag (zip lock bag, ≅ 0,1mm of thickness) covered with aluminium paper.

2.6. Chemical composition of the prepared cookies

2.6.1. Nutritional value

The pulverized samples were analysed according to the AOAC procedures in terms of macronutrients (moisture, proteins, fat, carbohydrates and ash) (AOAC, 2010). The macro-Kjeldahl method was used to estimate the crude protein content (Nx5.70). To determine the crude fat content, a Soxhlet extraction with petroleum ether was performed. Incineration at 600 ± 15 °C was used to measure ash content. Total carbohydrates were calculated by difference, and the energetic value calculated as: Energy (kcal) = 4× (g protein + g carbohydrate) + 9× (g fat).

2.6.2. Soluble sugars

A HPLC coupled to a refraction index (RI) detector (Knauer, Smartline system 1000, Berlin, Germany) was used to determine soluble sugars, using the internal standard method (IS, melezitose, Sigma-Aldrich, St. Louis, MO, USA), as previously described by Barros, Pereira, Calhelha et al. (2013). The mobile phase was a mixture of acetonitrile:water (70:30 v/v, acetonitrile HPLC-grade, Lab-Scan, Lisbon, Portugal), and separation was achieved using a Eurospher 100–5 NH_2 column (4.6 × 250 mm, 5 μm, Knauer). The results were recorded and processed using Clarity 2.4 software (DataApex, Prague, Czech Republic).

2.6.3. Tocopherols

Tocopherols determination followed the procedure previously described by Barros, Pereira, Calhelha et al. (2013). Briefly, the method comprises a HPLC system (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, USA) programmed for excitation at 290 nm and emission at 330 nm, using the IS method (tocol, Matreya, Pleasant Gap, PA, USA) for quantification. The mobile phase was a mixture of hexane:ethyl acetate (70:30, v/v, hexane and ethyl acetate HPLC-grade, Lab-Scan, Lisbon, Portugal), and chromatographic separation was performed using a Polyamide II column (250 × 4.6 mm, 5 μm; YMC, Kyoto, Japan). The results were recorded and processed using Clarity 2.4 software (DataApex, Prague, Czech Republic).

2.6.4. Fatty acids

A gas chromatographer (DANI1000, Contone, Switzerland) equipped with a split/splitless injector, and a flame ionization detector (GC-FID at 260 °C) operating in the conditions described by Barros, Pereira, and Ferreira (2013) was chosen. The identification and quantification of fatty acids was achieved by comparing the relative retention times of fatty acid methyl esters (FAME) reference standard mixture, Sigma-Aldrich, St. Louis, MO, USA) standards. The results were recorded and processed using CSW 1.7 software (Data Apex 1.7, Prague, Czech Republic).

2.7. Stability of the prepared cookie formulations

2.7.1. Physical parameters

2.7.1.1. Texture. A Stable Micro Systems (Vienna Court, Godalming UK) TA.XT) Plus Texture Analyser with a 30 Kg load cell, using the P/45 45 mm aluminium cylinder probe was used to carry out the texture analysis. A Texture Profile Analysis (TPA) was performed on the samples using a 5 mm/s as the pre- and post-test speed, and 3 mm/s as the test speed. The target mode was set to "strain" at 25% strain level for 5 consecutive seconds, while the trigger was set to "force" with measurement starting at 50 g of force. After the analysis, a macro was performed to measure various dimensions of texture, namely hardness, adhesiveness, springiness, cohesiveness, chewiness and resilience. The texture results were achieved through the Exponent program, proprietary of Stable Micro Systems.

2.7.1.2. Moisture. The cookie samples (2 g) were put in the metal plate and placed in a moisture analyzer (Adam Equipment, PMB 163). This equipment increases the temperature gradually to 105 °C to force moisture to evaporate from the food sample. When the weight achieves a constant value, i.e. no evaporation is detected, the sample is weighed a second time. The results were obtained using the following equation: % Moisture = (mi-mf)/mi × 100. Where mi is the initial weight and mf is the weight after reaching a constant weight.

2.7.1.3. Colour analysis. The external colour was measured in three different points of the cookies for the various storage times. Colour was on the exterior and interior of the cookie, as well as on the powder of ground cookies. This assay was performed with a portable CR400 colourimeter from Konica Minolta (Chiyoda, Tokyo, Japan) with the D65 illuminant, a standard illuminant defined by the International Commission on Illumination (CIE) which represents the midday light in Europe (daylight illuminant). The CIE L* a* b* colour space of 1976 was used, with L* representing lightness, a* representing redness (red-green), and b* representing yellowness (yellow-blue), with a 10° observation angle and 8 mm aperture.

2.7.2. Microbiological analysis

The final cookies were analysed for their stability regarding microbial growth control over the shelf life. Briefly, 1 g of the cookie powder was mixed with 9 mL of peptone water (PW, Liofilchem, Italy) and, from this suspension, serial decimal dilutions were prepared until achieving 10^{-3}. Afterwards, different counts were performed:

Aerobic plate count (total viable count; ISO 4833-2:2013: 1 mL of the prepared suspensions were inoculated in 15 mL of melted PCA (plate count agar, Liofilchem, Italy) (kept at 50 °C in a water bath or incubator), using the pour plate technique, in duplicate (LOQ = 1 log UFC/g). The plates were homogenized and left to solidify. The plates were then incubated at 30 °C for 72 h, in a reversed position. The counting was performed only in the plates presenting between 15 and 300 colonies.

Coliforms (and E. coli; ISO 4832:2006): 1 mL of the prepared suspensions were inoculated in 15 mL of melted VRBLA (violet red bile lactose agar, Liofilchem, Italy) (kept at 50 °C in a water bath or incubator), using the pour plate technique, in duplicate (LOQ = 1 log

UFC/g). The plates were homogenized and left to solidify. On the top of the medium, a top layer of 4 mL of VRBLA was poured, and it was left to solidify. Afterwards the plates were incubated at 30 °C for 48 h, in a reversed position. The counting was performed only in the plates presenting between 10 and 150 colonies.

Yeasts and Moulds (ISO 21527-1/2:2008): 0.2 mL of the prepared suspensions were pipetted onto a plate containing 15 mL of DRBC (dichloran rose bengal chloramphenicol, Liofilchem, Italy) by the spread plate technique, in duplicate (LOQ = 1.7 log UFC/g). The plates were further incubated at 25 °C for 5 days, in the upright position. The counting was performed in the plates having less than 150 colonies; the count of yeast and mould colonies was performed separately after 2 and 5 days of incubation, respectively.

Bacillus cereus (ISO 7932:2004). 0.2 mL of the prepared suspensions were pipetted onto a plate containing 15 mL of MYP (mannitol yolk polymyxin, Liofilchem, Italy) using the spread plate technique, in duplicate (LOQ = 1.7 log UFC/g). The plates were incubated at 30 °C for 24–48 h, in reversed position. The counting was performed in the plates showing between 10 and 150 colonies.

The microbial load of the different cookies was assessed after their preparation (t0) and after 30 days of storage.

2.8. Statistical analysis

Throughout the manuscript, all data is expressed as mean ± standard deviation. For the betacyanin's characterization of the two different natural extracts, an ANOVA was used followed by a Tukey's test to classify the differences among the three samples. If only two samples showed specific betacyanin compounds, the differences were sought by means of a simple Student's T-Test, using a significance level of 0.05 for both statistical analysis. For the cookie characterization, a two-way ANOVA with type III sums of squares using the SPSS Software, version 25. This multivariate general linear model treats the two factors, storage time (ST) and colourant type (CT) as independent, thus allowing the effect of each one to be analyzed independently, providing more insight to their contribution towards the outcome. If a significant interaction ($p < 0.05$) was recorded among the two factors (ST × CT), these were evaluated simultaneously, and some general conclusions and tendencies were extracted from the estimated marginal means (EMM) plots. If a significant interaction was not detected ($p > 0.05$), each factor was classified independently using a Tukey's multiple comparison test when the means were homoscedastic, and a Tamhane's T2 for non-homoscedastic samples. Homoscedasticity was evaluated using a Levene's test. All analyses were carried out using a significance level of 0.05.

3. Results and discussion

Cookies are bakery products of great commercial interest considering their production, commercialization and consumption characteristics, high demand, relatively long shelf life and good acceptability, particularly by children. Knowing the optimal proportions, and the impact of each ingredient in shape, appearance and product acceptance is very important, as demonstrated by Chin (1988). Therefore, several studies focusing on the improvement of this type of product, either by changing some of its ingredients, by using healthier alternatives, such as flour (Bassinello et al., 2011; Cheng & Bhat, 2016; Kaur, Singh, & Kaur, 2017), sugars (Aggarwal, Sabikhi, & Sathish Kumar, 2016), fibre supplementing (Baumgartner, Özkaya, Saka, & Özkaya, 2018; Galla, Pamidighantam, Karakala, Gurusiddaiah, & Akula, 2016; Mudgil, Barak, & Khatkar, 2017), or bioactive compounds (Infante et al., 2017) have been conducted. This trend can be justified by the growing appetence for natural additives, in response to the increasing need of industry, consumers, and to access the possibility to include these new product formulations in the functional product line.

The main objective of this work was to understand the influence of different colourants, namely a commercial one, E162, and another extracted from natural sources and dried in two different ways (lyophilization and spray-drying) all compared to a control sample, without any colouring. The influence of the colourants was analysed in different parameters, namely microbial load, nutritional profile, tocopherols presence, texture and exterior and interior colour.

3.1. Drying processes

Two different techniques were used to dry the betacyanin extracts, lyophilization and spray-drying. The spray-drying process had a yield of 20% due to inherent equipment losses. In the lyophilization process, a non-powdered and heterogeneous sample was obtained due to the presence of interfering molecules. With the spray-drying technique, a homogeneous, and shiny powder without agglomeration was produced due to the presence of maltodextrin. Even having a lower yield, the spray-drying technique provided an extract with better appearance and homogeneity. The main objective of using these two techniques was to evaluate which of them allows higher stability of the betacyanins and enables the obtaining of a homogeneous extract.

3.2. Betacyanins quantification and identification

Table 1 presents the peak characteristics (retention time, wavelength of maximum absorption and mass spectral data), tentative identification and quantification (mg/g of extract) of the betacyanin compounds present in the commercial colourant, lyophilized or spray dried extract of *G. globosa* flowers. Eleven different compounds were tentatively identified in the three samples. The tentative identification of the betacyanin extracts profile was performed following several descriptions already published by other authors. For *G. globosa* samples the tentative identification of peaks 3 ($[M+H]^+$ at *m/z* 551, gomphrenin I), 4 ($[M+H]^+$ at *m/z* 551, isogomphrenin I), 7 ($[M+H]^+$ at *m/z* 727, *cis*-Isomer of gomphrenin III), 8 ($[M+H]^+$ at *m/z* 727, *cis*-Isomer of isogomphrenin III), 9 ($[M+H]^+$ at *m/z* 697, gomphrenin II), 10 ($[M+H]^+$ at *m/z* 727, gomphrenin III), and 11 ($[M+H]^+$ at *m/z* 727, isogomphrenin III) was performed using the previously description made by Roriz, Barros, Carvalho, Santos-Buelga, and Ferreira (2014) of the same *G. globosa* sample, but also following the description made by Kugler, Stintzing, and Carle (2007) in crude extracts, Ferreres, Gil-Izquierdo, Valentão, and Andrade (2011) in aqueous extracts, and Cai, Xing, Sun, and Corke (2006) in hydroethanolic extracts of *G. globosa*.

The profile of the commercial extract obtained from *B. vulgaris*, as can be seen in Table 1, is completely different from *G. globosa*. Peaks 1 ($[M+H]^+$ at *m/z* 551, betanin), 2 ($[M+H]^+$ at *m/z* 551, isobetanin), 5 ($[M+H]^+$ at *m/z* 549, unknown compound) and 6 ($[M+H]^+$ at *m/z* 549, neobetanin), have been previously identified in beetroot juice (except for peak 5), being tentatively identified following the descriptions made by Nemzer et al. (2011). The presence of orange neobetanin pigments in *B. vulgaris* sample can also be a determining factor for the colorimetric difference between this commercial extract and the ones analyzed in this manuscript. The amount of betacyanins present in the commercial sample of beetroot is four-fold lower (39.36 ± 0.01 mg/g of extract) than in globe amaranth samples (161.6 ± 3.4 and 134 ± 2 mg/g of extract in the lyophilized and spray-dried sample, respectively), thus revealing that these extracts are excellent sources of these type of compounds. The large quantity obtained in the globe amaranth samples is mainly due to the presence of gomphrenin II (61 ± 2 and 45.25 ± 0.04 mg/g of extract) both in lyophilized and spray-dried samples, respectively. Between the two extracts (lyophilization and spray-drying), significant differences were detected, with lyophilization revealing the highest amount of total betacyanins, although the spraydrying extract showed a more intense coloration (Fig. 2). A more in-depth study should be carried out to uncover if the observed differences in the total amount of betacyanins justifies the expenses that the spray-drying technique implies.

Table 1
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification (mg/g of extract) of the betacyanins compounds present in the three samples studied (mean ± SD).

Peak	Rt	λmax (nm)	$[M-H]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	C	L	Sd	*t*-Studentstest *p*-value
1	17.26	533	551	389(1 0 0),345(5),150(5)	betanidin-5-*O*-*β*-glucoside (Betanin)	19.3 ± 0.2[c]	nd	nd	–
2	18.64	530	551	389(1 0 0),345(6),150(5)	Isobetanidin-5-*O*-*β*-glucoside (Isobetanin)	20.1 ± 0.2	nd	nd	–
3	19.63	533	551	389(1 0 0),345(6),150(7)	Gomphrenin I	nd	6.6 ± 0.1	6.9 ± 0.1	0.010
4	20.78	531	551	389(1 0 0),345(6),150(7)	Isogomphrenin I	nd	3.2 ± 0.1	3.3 ± 0.1	0.009
5	21.96	544	549	387(1 0 0)	Unknown compound	nq	nd	nd	–
6	22.65	544	465	387(1 0 0)	14,15-Dehydrobetanin (Neobetanin)	5.1 ± 0.1	nd	nd	–
7	30.11	544	727	551(52),389(1 0 0)	*cis*-Isomer of gomphrenin III	nd	31 ± 1	42 ± 1	< 0.001
8	31.19	542	727	551(49),389(1 0 0)	*cis*-Isomer of isogomphrenin III	nd	23.2 ± 0.1	16 ± 1	< 0.001
9	30.47	544	697	551(38),389(1 0 0)	Betanidin-6-*O*-(6′-*O*-*trans*-4-coumaroyl)-*β*-glucoside (gomphrenin II)	nd	61 ± 2	45.25 ± 0.04	< 0.001
10	31.67	545	727	551(60),389(1 0 0)	Betanidin-6-*O*-(6′-*O*-*trans*-feruloyl)-*β*-glucoside (gomphrenin III)	nd	19 ± 1	19.8 ± 0.2	0.030
11	32.11	543	727	551(54),389(1 0 0)	Isobetanidin-6-*O*-(6′-*O*-*trans*-feruloyl)-*β*-glucoside (isogomphrenin III)	nd	17.2 ± 0.2	nd	–
					Total	39.36 ± 0.01[c]	161.6 ± 3.4[a]	134 ± 2[b]	–

In each row, different letters mean significant differences. Classification of samples was carried out with a Tukey's post-hoc test for polyphenols present in all three samples, and a Student's T-test for polyphenols detected in only two samples. Nd – not detected. Nq – not quantifiable. C – commercial colourant; L – lyophilized extract; Sd – spray dried extract. Standard calibration curve used: Gomphrenin III ($y = 14670x - 19725$, $R^2 = 0.9997$).

3.3. Antioxidant activity

The best result was sought for the lyophilised extract of *G. globosa*, with an IC_{50} of 80 ± 2 μg/mL, followed by the spray-dried extract, with an IC_{50} of 176 ± 11 μg/mL, which was significantly different. These values translate the extract concentration required to protect 50% of the erythrocyte population from the haemolytic action caused by the oxidative agent, AAPH, for 60 min. Thus, the lower the IC_{50} value, the higher the antihaemolytic activity of the tested extracts. In turn, a higher concentration (699 ± 12 μg/mL) of the commercial colourant was required to protect the erythrocytes population during the same time period. Trolox, the used positive control, was more efficient in protecting the erythrocyte membranes than the tested extracts, with an IC_{50} value of 19 ± 1 μg/mL. However, trolox is a pure compound while the extracts are complex mixtures of different compounds with or without antioxidant activity. This *ex vivo* erythrocyte system offers test conditions close to *in vivo* since the oxidant AAPH generates peroxyl radicals in the *in vitro* system, which are also found in the human body (Takebayashi, Iwahashi, Ishimi, & Tai, 2012). These radicals are formed from the thermal decomposition of AAPH and attack the erythrocytes membrane, eventually causing its lysis. As a consequence, lipophilic radicals are generated through peroxidation phenomena, which also attack the polyunsaturated fatty acid-rich membranes. Overall, these results highlight the higher antioxidant capacity of the *G. globosa* extracts compared to the tested commercial colourant.

3.4. Chemical composition of the prepared cookie formulations

The two factors varying in this analysis were, the colourant type (CT) and storage time (ST), that, through a simple analysis of variance would not be correctly analysed due to the possibility of the combined effect of them to the outcome. Thus, a 2-way analysis of variance was used, allowing for and individual assessment of each parameter individually. In this way, Tables 2–4 are divided into two sections, the upper one with the different ST, ranging from 0 to 30 days, and the lower one representing the TC (control, commercial, lyophilized and spray dried). For each tested day in the upper section, all tested colourant types are included, and for each tested colourant in the lower section, all storage times are included. This type of representation and results interpretation allows for the aforementioned individual assessment of each parameter. Thus, the standard deviations should not be regarded as the accuracy of one individual analysis, but rather of a range of variation for the non-fixed parameter (CT or ST). If a significant interaction between these parameters is detected, by having the *p*-value of CT × ST lower than 0.05, no multiple comparisons can be extracted, meaning that both parameters (CT and ST) had significant contributions for the changes, which only allows tendencies to be extracted from the Estimated Marginal Means (EMM) plots. If inversely, the *p*-value of CT × ST is higher than 0.05, each parameter is analyzed individually.

3.4.1. Nutritional profile

The centesimal profile, expressed in g/100 of dry weight, is listed in the left section of Table 2. The profile included fat, protein, ash and carbohydrate content (calculated by difference) and energy. The nutrients detected in the highest quantity were the carbohydrates (although they are comprised of fibres present in the flour, and also the added sucrose), followed by fat and protein. A significant interaction among the two analysed parameters (storage time and colourant type) was detected (ST × CT < 0.05), which hindered any individual assessment of the individual contribution of each parameter. Still, given the low amount of water in the cookies, significant variations in the nutritional profile were not expected.

Table 2
Nutritional profile of the different biscuit types along the 30-day storage time, represented in g/100 g of dry weight (dw) (left section), as well as the different dimensions of texture (right section).

		Fat	Proteins	Ash	Carbohydrates	EnergyKcal	Hardness (g)	Adhesiveness(g.sec)	Springiness(%)	Cohesiveness (%)	Chewiness	Resilience(%)
Storage time (ST)	0 days	23 ± 1	5.2 ± 0.2	1.8 ± 0.1	70 ± 1	509 ± 5	15688 ± 4953	−29 ± 28	0.49 ± 0.09	0.30 ± 0.05	2727 ± 1664	0.16 ± 0.04
	7 days	25 ± 1	5.5 ± 0.3	1.79 ± 0.09	67.71 ± 0.09	519 ± 8	8442 ± 3640	−4 ± 4	0.8 ± 0.1	0.54 ± 0.07	1881 ± 740	0.23 ± 0.04
	15 days	23.4 ± 0.7	5.3 ± 0.3	1.7 ± 0.2	69.6 ± 0.7	510 ± 3	6656 ± 977	−3 ± 1	0.69 ± 0.05	0.39 ± 0.07	1860 ± 579	0.220 ± 0.01
	30 days	24.7 ± 0.7	5.3 ± 0.3	1.7 ± 0.1	68.3 ± 0.7	517 ± 3	3394 ± 1310	−22 ± 22	0.6 ± 0.1	0.3 ± 0.1	1394 ± 813	0.2 ± 0.1
p-value (n = 5)	Tukey's HSD test	< 0.001	< 0.001	0.022	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Colourant Type (CT)	Control	25 ± 1	5.2 ± 0.2	1.8 ± 0.1	68 ± 1	517 ± 8	7836 ± 4286	−16 ± 24	0.5 ± 0.1	0.4 ± 0.1	1337 ± 648	0.3 ± 0.1
	Commercial	24.6 ± 0.7	5.3 ± 0.3	1.81 ± 0.08	68.2 ± 0.9	516 ± 4	9531 ± 6545	−8 ± 5	0.67 ± 0.09	0.44 ± 0.08	2702 ± 1304	0.2 ± 0.1
	Lyophilized	24 ± 1	5.5 ± 0.1	1.7 ± 0.2	69 ± 1	512 ± 7	9092 ± 6796	−8 ± 8	0.8 ± 0.2	0.32 ± 0.09	2079 ± 1292	0.22 ± 0.05
	Spray Dried	23.2 ± 0.8	5.3 ± 0.3	1.7 ± 0.1	69.4 ± 0.7	509 ± 4	7721 ± 3986	−27 ± 30	0.6 ± 0.1	0.3 ± 0.1	1743 ± 667	0.23 ± 0.09
p-value (n = 20)	Tukey's HSD test	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
ST × CT (n = 80)	*p*-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

The presented standard deviations were calculated from results obtained under different operational conditions. Thus, they should not be regarded as a measure of precision, rather as a range of values.

3.4.2. Soluble sugars and tocopherols

Sucrose was the only detected sugar by HPLC-RI, and is represented in g/100 g of dry weight on Table 3 Furthermore, tocopherols, isoforms of vitamin E were detected through HPLC coupled to a fluorescence detector, and are also presented in Table 3, expressed in mg/100 g of dry weight. Sucrose was detected since it is the sugar added to the recipe and thus it is expected to be found at a high amount, ranging from 47 to 51 g/100 g. Also, it was expected that after 30 days of storage, sucrose would breakdown to fructose and glucose, and despite this having occurred, the detected amounts were residual, probably due to the very low content of water found in the cookies that halted the breakdown. A significant interaction was detected for sucrose, and thus, both parameters promoted the verified changes, which due to the low moisture in the cookies was not significant. Regarding tocopherols, only three of the four isoforms were detected, namely α-, β-, and δ-tocopherol. The most abundant isoform detected was α-tocopherol, followed by δ-tocopherol, and finally γ-tocopherol. Vitamin E could be present in any of the three main ingredients, namely butter, sunflower oil and flour, although none of the labels stated the amounts of vitamin E. Still, and appreciable quantity of tocopherols was found, amounting to 39 mg/100 g. Moreover, and once again, a significant interaction was verified, and little to no variations were detected for tocopherols, once again due to residual amounts of water and the fact that the cookies did not undergo lipidic oxidation, which would reduce the content of tocopherols over the storage time.

3.4.3. Individual fatty acids

Table 3 also represents the individual fatty acids composition, namely monounsaturated (MUFA), polyunsaturated (PUFA) and saturated fatty acids (SFA), as detected through GC-FID along the cookie's storage time of 30 days. Values are expressed as relative percentages. Although a higher number of individual fatty acids were detected, only the ones representing at least, 1% of the total amount are shown in Table 3. The most abundant individual fatty acid was palmitic acid (C16:0), followed by oleic (C18:1) and linoleic acid (C18:2). This profile is consistent with the used ingredients, being mainly composed by butter, sunflower oil and flour. In terms of the fatty acid groups, the SFA prevailed over the unsaturated ones, with roughly 60% of the total amount, while the MUFA reached an average of 24%, and PUFA only 12–13%. As can be inferred by the *p*-value of the interaction, *p*-value < 0.05, there was a significant interaction between the two parameters, CT and ST, in terms of the variation of the fatty acids. Furthermore, the EMM did not show any general tendencies, but, observing the variations of the fatty acids, it is clear that there was no major variation along the storage time and the influence of the colourant was not enough to drastically change the fatty acid profile. These results were expected mainly because the cookies had very low water quantities and 30 days was not enough time to promote an oxidative cascade in the lipid fraction.

3.5. Cookie stability over storage time

3.5.1. Physical parameters

The right section of Table 2 displays the different dimensions of the texture analysis, including hardness, adhesiveness, springiness, cohesiveness, chewiness and resilience. Once again, the overall changes in texture were a result of a significative interaction between the colourant type and storage time. Still, over time, the cookie hardness decreased, probably due to retrogradation, in which moisture is captured from the surrounding environment, often softening the biscuit. Furthermore, these variations could also be explained by the low values of glutein which, in high amounts result in a more stable dough with lower hardness changes over time (Barak, Mudgil, & Khatkar, 2013). Adhesiveness, springiness, cohesiveness and resilience did not undergo drastic changes, and the ones registered were a product of the interaction of the storage time and colourant type, showing a consistency

Table 3
Sucrose (g/100 g dw), tocopherol isoforms (mg/100 g dw) (left section) and individual fatty acids, MUFA, PUFA and SFA of the different cookies along the 30 days of storage time, represented as a relative percentage of themselves (right section).

		Sucrose	α-tocopherol	β-tocopherol	δ-tocopherol	Total tocopherols	C6:0	C8:0	C10:0
Storage time (ST)	0 days	48 ± 3	29.5 ± 0.7	2.0 ± 0.2	7.9 ± 0.7	39.5 ± 0.1	2.9 ± 0.2	1.42 ± 0.08	2.9 ± 0.1
	7 days	50 ± 1	28.2 ± 0.8	2.3 ± 0.3	8.8 ± 0.9	39.4 ± 0.1	3.4 ± 0.6	1.4 ± 0.1	2.7 ± 0.2
	15 days	48 ± 2	29.2 ± 0.8	2.0 ± 0.1	8.4 ± 0.6	39.6 ± 0.2	3.3 ± 0.3	1.5 ± 0.1	2.8 ± 0.1
	30 days	48 ± 2	30 ± 1	1.9 ± 0.2	7 ± 1	39.6 ± 0.2	3.1 ± 0.2	1.4 ± 0.1	2.6 ± 0.1
p-value (n = 5)	Tukey's HSD test	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Colourant Type (CT)	Control	51 ± 2	29.8 ± 0.9	1.9 ± 0.2	7.9 ± 0.7	39.6 ± 02	3.0 ± 0.2	1.4 ± 0.1	2.7 ± 0.2
	Commercial	49 ± 2	29.2 ± 0.4	1.97 ± 0.07	8.4 ± 0.3	39.5 ± 0.1	3.2 ± 0.3	1.44 ± 0.08	2.79 ± 0.07
	Lyophilized	47 ± 2	28.1 ± 08	2.20 ± 0.09	9.1 ± 0.6	39.4 ± 0.1	3.2 ± 0.2	1.4 ± 0.1	28 ± 0.3
	Spray Dried	48 ± 2	30 ± 1	2.2 ± 0.4	7 ± 1	39.6 ± 0.2	3.3 ± 0.7	1.4 ± 0.1	2.7 ± 0.1
p-value (n = 20)	Tukey's HSD test	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
ST × CT (n = 80)	*p*-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1n9c	C18:2n6c	SFA	MUFA	PUFA
Storage time (ST)	3.9 ± 0.1	10.7 ± 0.3	30.2 ± 0.8	1.30 ± 0.02	10.3 ± 0.3	22.0 ± 0.4	12 ± 1	64 ± 1	24.1 ± 0.4	12.0 ± 0.9
	3.6 ± 0.1	10.1 ± 0.3	29.2 ± 0.4	1.24 ± 0.03	10.1 ± 0.4	23 ± 1	12.8 ± 0.7	61 ± 2	25 ± 1	13.2 ± 0.7
	3.55 ± 0.07	10.10 ± 0.1	29.7 ± 0.4	1.24 ± 0.04	10.6 ± 0.2	22.3 ± 0.2	12.4 ± 0.8	62.8 ± 0.9	24.4 ± 0.2	12.8 ± 0.8
	3.6 ± 0.2	10.2 ± 0.3	29.5 ± 0.4	1.29 ± 0.03	10.6 ± 0.1	22.6 ± 0.2	12.3 ± 0.9	62 ± 1	24.8 ± 0.2	12.7 ± 0.9
p-value (n = 5)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Colourant Type (CT)	3.5 ± 0.1	10.0 ± 0.1	29.1 ± 0.2	1.250 ± 0.02	10.3 ± 0.2	22.7 ± 0.2	13.4 ± 0.2	61.4 ± 0.4	24.7 ± 0.2	13.8 ± 0.2
	3.7 ± 0.2	10.2 ± 0.3	29.8 ± 0.3	1.28 ± 0.03	10.7 ± 0.1	22.5 ± 0.4	11.9 ± 0.2	63.1 ± 0.4	24.6 ± 0.4	12.3 ± 0.1
	3.7 ± 0.2	10.4 ± 0.4	29.9 ± 0.6	1.26 ± 0.06	10.4 ± 0.5	23 ± 1	11 ± 1	63 ± 2	25 ± 1	12 ± 1
	3.7 ± 0.2	10.4 ± 0.3	29.9 ± 0.9	1.28 ± 0.02	10.3 ± 0.3	22.0 ± 0.5	12.3 ± 0.7	63 ± 1	24.1 ± 0.6	12.7 ± 0.7
p-value (n = 20)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
ST × CT (n = 80)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

C6:0 – Caproic acid; C8:0 – Caprylic acid; C10:0 – Capric acid; C12:0 – Lauric acid; C14:0 – Myristic acid; C16:0 – Palmitic acid; C16:1 – Palmitoleic acid; C18:0 – Stearic acid; C18:1n9 – Oleic acid; C18:2n6 – Linoleic acid. SFA: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids. The presented standard deviations were calculated from results obtained under different operational conditions. Thus, they should not be regarded as a measure of precision, rather as a range of values.

Table 4
Representation of the external (Cookies), internal (Interior) and the biscuit powder (Milled Sample) colours, according to the L*, a*, b* colour space.

		Cookies			Interior			Milled Sample		
		L*	a*	b*	L*	a*	b*	L*	a*	b*
Storage time (ST)	0 days	62 ± 7	18 ± 11	15 ± 9	58 ± 9	14 ± 8	12 ± 8	67 ± 8	16 ± 10	16 ± 9
	7 days	62 ± 9	17 ± 11	14 ± 10	60 ± 7	14 ± 8	12 ± 8	66 ± 9	16 ± 10	16 ± 10
	15 days	62 ± 9	17 ± 11	15 ± 10	61 ± 7	14 ± 8	13 ± 8	67 ± 8	17 ± 10	17 ± 9
	30 days	62 ± 8	17 ± 11	16 ± 9	64 ± 7	14 ± 9	12 ± 7	68 ± 8	17 ± 11	17 ± 9
p-value (n = 5)	Tukey's HSD test	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Colourant Type (CT)	Control	75 ± 1	−1.0 ± 0.2	28.7 ± 0.6	73 ± 2	−0.2 ± 0.5	22.9 ± 0.5	82.3 ± 0.8	−0.8 ± 0.2	29.1 ± 0.5
	Commercial	60.7 ± 0.6	23.2 ± 0.7	18.1 ± 0.5	59 ± 2	18.7 ± 0.8	15.6 ± 0.7	64.8 ± 0.5	22.1 ± 0.9	21 ± 1
	Lyophilized	56.3 ± 0.6	22.1 ± 0.2	8 ± 1	56 ± 2	17.5 ± 0.4	7.4 ± 0.4	61 ± 1	21.5 ± 0.8	11.0 ± 0.7
	Spray Dried	56 ± 1	25.5 ± 0.4	4.3 ± 0.4	55 ± 4	20.5 ± 0.3	2.9 ± 0.4	61.2 ± 0.9	24.8 ± 0.6	5.9 ± 0.6
p-value (n = 20)	Tukey's HSD test	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
ST × CT (n = 80)	*p*-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

The presented standard deviations were calculated from results obtained under different operational conditions. Thus, they should not be regarded as a measure of precision, rather as a range of values.

along the storage time. Chewiness, which is defined as the energy required to masticate the food, also decreased over time, in line with the decrease of hardness, providing that softer foods require less energy to masticate. In this way, and although both parameters (CT and ST) contributed, the cookies became softer overtime, and their chewiness decreased in line with this trend.

Cookies are snacks that, beyond being appealing to the palate, also have to be appealing to the eye of the consumer, hence the need to colour them or to intensify their natural colours. Thus, the colour analysis is of crucial importance, and the resistance of colourants to oven cooking and storage time is of utmost importance for their success in the industry. To obtain a better understanding of the colouring capacity of these colourants, the colour, measured with a portable colourimeter, was analysed on the outer part of the cookies, but also in the internal one, and finally in their powder after milling. This procedure allows some insights into the effects of cooking and storage time, both on the outer part of the cookies, but also the inner section. Colour was analysed using the CIELab colour space, that measures coordinates of L* (lightness, variation between −100 and +100, black to white), a* (greenness and redness, variation between −100 and +100, red to green), and b* (yellowness/blueness, variation between −100 and +100, yellow to blue). There was a significative interaction between storage time and colourant type in Table 4, although some tendencies could be extracted from the EMM plots. This table shows the L*, a* and b* coordinates of the external and internal colour of cookies, as well as their powder.

The main objective of this work was to achieve a pink colouration on the cookies, the ideal coordinates are an average of 0 for L*, +100

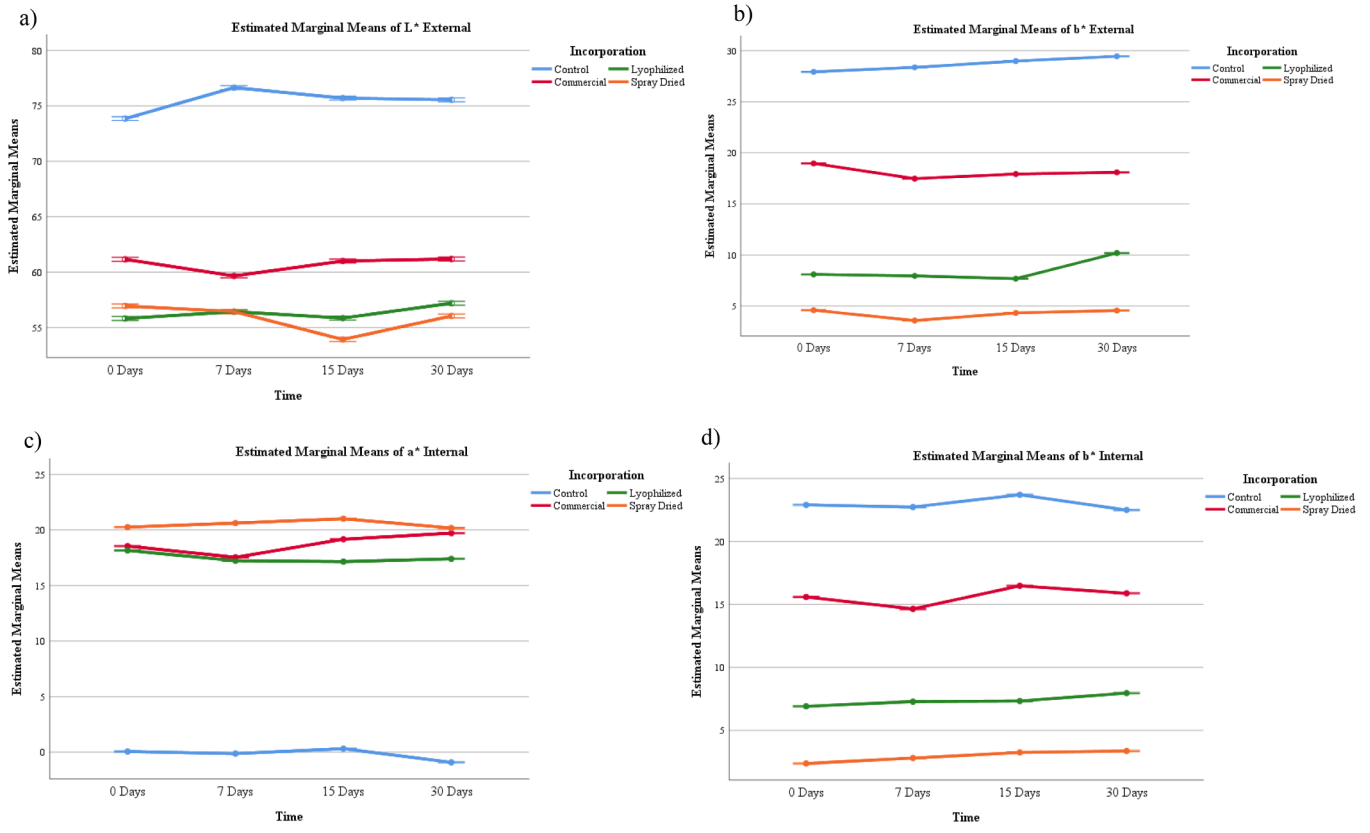

Fig. 1. EMM plots of the external and internal colour of the cookies during the 30-day storage time.

for a* and 0 for b*. Fig. 1 shows the different coordinates of the external and internal colour for the different incorporated cookies along the 30-days of storage time. It is clear that, in line with other parameters, the variation in colour is quite low due to the low water content and the absence of oxidation during the storage time; still, some changes did occur. As stated above, to achieve the pink colouration, the lightness (L*) should be near 0, and thus, both the lyophilized and spray dried cookies showed the lowest values throughout the storage time (Fig. 1a) for the external colour. In terms of b*, where the desired amount is 0, both cookies with spray-dried extract and lyophilized of *G. globosa* showed similar values, thus proving a more homogenous pink colour (Fig. 1b). The same trend was noticed for the colours measured in the interior samples, as can be seen in Fig. 1c and d, that displays the EMM plots of a* and b*. Fig. 2a shows the colour of the three different colourants used by converting the L*, a* and b* coordinates to RGB colours. A darker colour is shown for the commercial sample and lyophilized extract, showing a high similarity, while the lyophilized extract is lighter and shows a color resembling violet. Fig. 2b shows the overall colour of the cookies themselves by averaging the colours during the 30 days of storage. The external colour of the natural coloured cookies was more consistent with a pink, while the commercial samples showed a shift to orange. The deeper pink colour can be observed for all cookies added with the spray-dried extract of *G. globosa*, followed by the lyophilized form, especially in the internal section of the cookie. The orange colour, detected in commercial sample does not fit the pink colouration intended for the enhancement of the biscuit's appearance, thus representing an opportunity for these natural colourants to fill a void in the food industry in terms of food colouring.

3.5.2. Microbiological analysis

To guarantee the safety of the developed colourants, the prepared cookies were analysed for their microbial counting over their shelf life (30 days), due to the risk that microorganisms could hinder the cookie's stability due to degradation. No microbial contamination was detected along the 30 days of analysis, meaning that the cooking process was able to eliminate possible microorganisms present in the flour or extracts.

4. Conclusions

The fact that the spray-drying samples showed a deeper pink coloration can be related to the protective effect of the spray-drying process, which seems to be effective for the betacyanins presented in the extracts. The enveloping of the extract with maltodextrin, contributed to this protection, and played an important role during the baking process. Inversely, the lyophilized extract and the commercial colourant did not achieve this protective effect conferred by maltodextrin, and thus, the color did not maintain the intended deep pink. However, the lyophilized extract seemed to lose at a lower extent the colour intensity when compared to the commercial sample (which is expected to resist high temperatures). This lyophilized extract can be considered a natural alternative, with a somewhat better performance than the commercial sample, probably associated with the extraction procedure that allowed a higher yield of coloring molecules, and also a comparably reduced extraction time. Furthermore, both drying techniques provided a higher stability over time, beyond presenting no degradation and no microbial count. Moreover, the incorporation did not significantly alter the chemical composition of the cookies, which is paramount for all food additives to be considered for these purposes. In terms of marketing viability, when comparing the lyophilized and spray-dried extracts, the lyophilized seem to show better results, provided that the pink intensity can be achieved by adding a higher amount of extract. Because the production costs of the lyophilized extract are considerably lower, the corresponding extract can be an interesting alternative to commercial colourants.

Fig. 2. Colours of the cookies, obtained from the L*, a*, b* coordinates, considering an average of the colours during all storage times.

CRediT authorship contribution statement

Custódio Lobo Roriz: Methodology, Investigation, Writing - original draft. **Sandrina A. Heleno:** Methodology, Writing - review & editing. **Márcio Carocho:** Methodology. **Paula Rodrigues:** Methodology. **José Pinela:** Methodology, Writing - original draft. **Maria Inês Dias:** Methodology. **Isabel P. Fernandes:** Methodology. **Maria Filomena Barreiro:** Methodology. **Patricia Morales:** Methodology, Writing - review & editing. **Lillian Barros:** Conceptualization, Methodology, Writing - review & editing. **Isabel C.F.R. Ferreira:** Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support by national funds FCT/MCTES to CIMO (UIDB/00690/2020); C. L. Roriz PhD's grant (SFRH/BD/117995/2016), L. Barros and Isabel P. Fernandes to FCT, P.I., through the institutional scientific employment program-contract for their contract and the individual scientific employment program-contract for S.A. Heleno's contract. The authors are also grateful to the European Regional Development Fund (ERDF) through the Regional Operational Program North 2020, within the scope of project Mobilizador Norte-01-0247-FEDER-024479: ValorNatural®. M. Carocho also acknowledges ValorNatural® for his contract. Finally, P. Morales is also grateful to UCM ALIMNOVA Research Group (GR105/18).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127178.

References

Aggarwal, D., Sabikhi, L., & Sathish Kumar, M. H. (2016). Formulation of reduced-calorie biscuits using artificial sweeteners and fat replacer with dairy-multigrain approach. *NFS Journal, 2*, 1–7. https://doi.org/10.1016/j.nfs.2015.10.001.

AOAC. (2010). Official Methods of Analysis of AOAC International 962.09. Association of Official Analysis Chemists International, (February), 2–4.

Barak, S., Mudgil, D., & Khatkar, B. S. (2013). Effect of composition of gluten proteins and dough rheological properties on the cookie-making quality. *British Food Journal, 115*(4), 564–574. https://doi.org/10.1108/00070701311317847.

Barros, L., Pereira, C., & Ferreira, I. C. F. R. (2013). Optimized analysis of organic acids in edible mushrooms from portugal by ultra fast liquid chromatography and photodiode array detection. *Food Analytical Methods, 6*(1), 309–316. https://doi.org/10.1007/s12161-012-9443-1.

Barros, L., Pereira, E., Calhelha, R. C., Dueñas, M., Carvalho, A. M., Santos-Buelga, C., & Ferreira, I. C. F. R. (2013). Bioactivity and chemical characterization in hydrophilic and lipophilic compounds of *Chenopodium ambrosioides* L. *Journal of Functional Foods, 5*(4), 1732–1740. https://doi.org/10.1016/j.jff.2013.07.019.

Bassinello, P. Z., de Freitas, D. G. C., Ascheri, J. L. R., Takeiti, C. Y., Carvalho, R. N., Koakuzu, S. N., & Carvalho, A. V. (2011). Characterization of cookies formulated with rice and black bean extruded flours. *Procedia Food Science, 1*, 1645–1652. https://doi.org/10.1016/j.profoo.2011.09.243.

Baumgartner, B., Özkaya, B., Saka, I., & Özkaya, H. (2018). Functional and physical properties of cookies enriched with dephytinized oat bran. *Journal of Cereal Science, 80*, 24–30. https://doi.org/10.1016/j.jcs.2018.01.011.

Cai, Y. Z., Xing, J., Sun, M., & Corke, H. (2006). Rapid identification of betacyanins from *Amaranthus tricolor*, *Gomphrena globosa*, and *Hylocereus polyrhizus* by matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry (MALDI-QIT-TOF MS). *Journal of Agricultural and Food Chemistry, 54*(18), 6520–6526. https://doi.org/10.1021/jf0609983.

Calogero, G., Yum, J.-H., Sinopoli, A., Di Marco, G., Grätzel, M., & Nazeeruddin, M. K. (2012). Anthocyanins and betalains as light-harvesting pigments for dye-sensitized solar cells. *Solar Energy, 86*(5), 1563–1575. https://doi.org/10.1016/j.solener.2012.02.018.

Carocho, M., Morales, P., & Ferreira, I. C. F. R. (2015). Natural food additives: Quo vadis? *Trends in Food Science & Technology, 45*(2), 284–295. https://doi.org/10.1016/j.tifs.2015.06.007.

Cheng, Y. F., & Bhat, R. (2016). Functional, physicochemical and sensory properties of novel cookies produced by utilizing underutilized jering (*Pithecellobium jiringa* Jack.) legume flour. *Food Bioscience, 14*, 54–61. https://doi.org/10.1016/j.fbio.2016.03.002.

Chin, L. (1988). Effect of ingredient ratio on the sweetness of cookies. *Journal of Home Economics of Japan, 39*(8), 889–893. https://doi.org/10.11428/jhej1987.39.889.

Corrêa, R. C. G., Garcia, J. A. A., Correa, V. G., Vieira, T. F., Bracht, A., & Peralta, R. M. (2019). Pigments and vitamins from plants as functional ingredients: Current trends and perspectives. *Advances in Food and Nutrition Research, 90*, 259–303. https://doi.org/10.1016/bs.afnr.2019.02.003.

Ferreres, F., Gil-Izquierdo, A., Valentão, P., & Andrade, P. B. (2011). Structural characterization of phenolics and betacyanins in *Gomphrena globosa* by high-performance liquid chromatography-diode array detection/electrospray ionization multi-stage mass spectrometry. *Rapid Communications in Mass Spectrometry, 25*(22), 3441–3446.

Galla, N. R., Pamidighantam, P. R., Karakala, B., Gurusiddaiah, M. R., & Akula, S. (2016). Nutritional, textural and sensory quality of biscuits supplemented with spinach (*Spinacia oleracea* L.). *International Journal of Gastronomy and Food Science, 7*(December 2016), 20–26. https://doi.org/10.1016/j.ijgfs.2016.12.003.

Infante, R. A., Natal, D. I. G., de Moreira, M. E. C., Bastiani, M. I. D., Chagas, C. G. O., Nutti, M. R., Queiróz, V. A. V., & Martino, H. S. D. (2017). Enriched sorghum cookies with biofortified sweet potato carotenoids have good acceptance and high iron bioavailability. *Journal of Functional Foods, 38*, 89–99. https://doi.org/10.1016/j.jff.2017.08.044.

Kaur, M., Singh, V., & Kaur, R. (2017). Effect of partial replacement of wheat flour with varying levels of flaxseed flour on physicochemical, antioxidant and sensory characteristics of cookies. *Bioactive Carbohydrates and Dietary Fibre, 9*(August 2016), 14–20. https://doi.org/10.1016/j.bcdf.2016.12.002.

Kugler, F., Stintzing, F. C., & Carle, R. (2007). Characterisation of betalain patterns of differently coloured inflorescences from *Gomphrena globosa* L. and *Bougainvillea* sp. by HPLC-DAD-ESI-MSn. *Analytical and Bioanalytical Chemistry, 387*(2), 637–648.

Kumar, N., Singh, A., Sharma, D. K., & Kishore, K. (2019). Toxicity of food additives. *Food Safety and Human Health*. https://doi.org/10.1016/b978-0-12-816333-7.00003-5.

Leong, H. Y., Ooi, C. W., Law, C. L., Julkifle, A. L., Pan, G. T., & Show, P. L. (2018). Investigation of betacyanins stability from peel and flesh of red-purple pitaya with food additives supplementation and pH treatments. *Lwt, 98*(February), 546–558. https://doi.org/10.1016/j.lwt.2018.09.021.

Lockowandt, L., Pinela, J., Roriz, C. L., Pereira, C., Abreu, R. M. V., Calhelha, R. C., ... Ferreira, I. C. F. R. (2019). Chemical features and bioactivities of cornflower (*Centaurea cyanus* L.) capitula: The blue flowers and the unexplored non-edible part. *Industrial Crops and Products, 128*(November 2018), 496–503. https://doi.org/10.1016/j.indcrop.2018.11.059.

Majerska, J., Michalska, A., & Figiel, A. (2019). A review of new directions in managing fruit and vegetable processing by-products. *Trends in Food Science and Technology, 88*(March), 207–219. https://doi.org/10.1016/j.tifs.2019.03.021.

Maqsood, S., Adiamo, O., Ahmad, M., & Mudgil, P. (2020). Bioactive compounds from date fruit and seed as potential nutraceutical and functional food ingredients. *Food Chemistry, 308*(September 2019), 125522. https://doi.org/10.1016/j.foodchem.2019.125522.

Martins, F. C. O. L., Sentanin, M. A., & De Souza, D. (2019). Analytical methods in food additives determination: Compounds with functional applications. *Food Chemistry, 272*(April 2018), 732–750. https://doi.org/10.1016/j.foodchem.2018.08.060.

Martins, N., Roriz, C. L., Morales, P., Barros, L., & Ferreira, I. C. F. R. (2016). Food colorants: Challenges, opportunities and current desires of agro-industries to ensure consumer expectations and regulatory practices. *Trends in Food Science & Technology, 52*, 1–15. https://doi.org/10.1016/j.tifs.2016.03.009.

Martins, N., Roriz, C. L., Morales, P., Barros, L., & Ferreira, I. C. F. R. (2017). Coloring attributes of betalains: A key emphasis on stability and future applications. *Food & Function, 8*(4), 19–23. https://doi.org/10.1039/C7FO00144D.

Melgar, B., Dias, M. I., Ciric, A., Sokovic, M., Garcia-Castello, E. M., Rodriguez-Lopez, A. D., ... Ferreira, I. C. R. F. (2017). By-product recovery of *Opuntia* spp. peels: Betalainic and phenolic profiles and bioactive properties. *Industrial Crops and Products, 107*(June), 353–359. https://doi.org/10.1016/j.indcrop.2017.06.011.

Molina, A. K., Vega, E. N., Pereira, C., Dias, M. I., Heleno, S. A., Rodrigues, P., ... Ferreira, I. C. F. R. (2019). Promising antioxidant and antimicrobial food colourants from *Lonicera caerulea* L. var. Kamtschatica. *Antioxidants, 8*(9), 1–18. https://doi.org/10.3390/antiox8090394.

Mudgil, D., Barak, S., & Khatkar, B. S. (2017). Cookie texture, spread ratio and sensory acceptability of cookies as a function of soluble dietary fiber, baking time and different water levels. *LWT – Food Science and Technology, 80*, 537–542. https://doi.org/10.1016/j.lwt.2017.03.009.

Nemzer, B., Pietrzkowski, Z., Spórna, A., Stalica, P., Thresher, W., Michałowski, T., & Wybraniec, S. (2011). Betalainic and nutritional profiles of pigment-enriched red beet root (*Beta vulgaris* L.) dried extracts. *Food Chemistry, 127*(1), 42–53. https://doi.org/10.1016/j.foodchem.2010.12.081.

Roriz, C. L., Barreira, J. C. M., Morales, P., Barros, L., & Ferreira, I. C. F. R. (2018). *Gomphrena globosa* L. as a novel source of food-grade betacyanins: Incorporation in ice-cream and comparison with beet-root extracts and commercial betalains. *Lwt, 92*(February), 101–107. https://doi.org/10.1016/j.lwt.2018.02.009.

Roriz, C. L., Barros, L., Carvalho, A. M., Santos-Buelga, C., & Ferreira, I. C. F. R. (2014). *Pterospartum tridentatum*, *Gomphrena globosa* and *Cymbopogon citratus*: A phytochemical study focused on antioxidant compounds. *Food Research International, 62*, 684–693. https://doi.org/10.1016/j.foodres.2014.04.036.

Roriz, C. L., Barros, L., Prieto, M. A., Barreiro, M. F., Morales, P., & Ferreira, I. C. F. R.

(2017). Modern extraction techniques optimized to extract betacyanins from *Gomphrena globosa* L. *Industrial Crops & Products, 105*(May), 29–40. https://doi.org/10.1016/j.indcrop.2017.05.008.

Roriz, C. L., Barros, L., Prieto, M. A., Morales, P., & Ferreira, I. C. F. R. (2017). Floral parts of *Gomphrena globosa* L. as a novel alternative source of betacyanins: Optimization of the extraction using response surface methodology. *Food Chemistry, 229*, 223–234. https://doi.org/10.1016/j.foodchem.2017.02.073.

Stich, E. (2016). Food Color and Coloring Food: Quality, Differentiation and Regulatory Requirements in the European Union and the United States. Quality, Differentiation and Regulatory Requirements in the European Union and the United States. In Handbook on Natural Pigments in Food and Beverages: Industrial Applications for Improving Food Color. https://doi.org/10.1016/B978-0-08-100371-8.00001-4.

Takebayashi, J., Iwahashi, N., Ishimi, Y., & Tai, A. (2012). Development of a simple 96-well plate method for evaluation of antioxidant activity based on the oxidative haemolysis inhibition assay (OxHLIA). *Food Chemistry, 134*(1), 606–610. https://doi.org/10.1016/j.foodchem.2012.02.086.

Tomaska, L. D., & Brooke-Taylor, S. (2014). Food Additives – General. In Encyclopedia of Food Safety (Vol. 2). https://doi.org/10.1016/B978-0-12-378612-8.00234-1.

Wu, S., Bao, Y., Wang, D., Wang, X., Liu, H., Li, Z., ... Guo, Q. (2020). Water-soluble complexes of orange pigments from *Monascus* sp. with HP-β-CD: Preparation, inclusion mechanism, and improved stability. *Journal of Molecular Liquids, 300*, 112212. https://doi.org/10.1016/j.molliq.2019.112212.

Zhang, D., Cheng, X., Sun, D., Ding, S., Cai, P., Yuan, L., ... Hu, Q. N. (2020). AdditiveChem: A comprehensive bioinformatics knowledge-base for food additive chemicals. *Food Chemistry, 308*(April 2019), 125519. https://doi.org/10.1016/j.foodchem.2019.125519.